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Induction of BIM, a Proapoptotic BH3-Only BCL-2 Family Member, Is Critical for Neuronal Apoptosis

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Summary

Sympathetic neuronal death induced by nerve growth factor (NGF) deprivation requires the macromolecular synthesis–dependent translocation of BAX from the cytosol to mitochondria and its subsequent integration into the mitochondrial outer membrane, followed by BAX-mediated cytochrome c (cyt c) release. The gene products triggering this process remain unknown. Here, we report that BIM, a member of the BH3-only proapoptotic subfamily of the BCL-2 protein family, is one such molecule. NGF withdrawal induced expression of BIM_{EL}, an integral mitochondrial membrane protein that functions upstream of (or in parallel with) the BAX/BCL-2 and caspase checkpoints. *Bim* deletion conferred protection against developmental and induced neuronal apoptosis in both central and peripheral populations, but only transiently, suggesting that BIM—and perhaps other BH3-only proteins—serve partially redundant functions upstream of BAX-mediated cyt c release.

Introduction

Programmed cell death (PCD) is an evolutionarily conserved and genetically regulated process that is critical to the development and maintenance of many tissues. Deficiencies in PCD underlie some forms of oncogenesis, whereas excessive cell death may contribute to several pathological conditions, including stroke, autoimmune disorders, and certain neurodegenerative diseases (Thompson,1995). Cells undergoing PCD exhibit morphological and biochemical changes characteristic of apoptosis, including cytoplasmic shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation (Kerr et al., 1972). Nonprofessional phagocytes eventually engulf the dying cells, thereby preventing an inflammatory response.

In mammals, signaling cascades culminating in apoptotic cell death can be divided into two broad categories: the "intrinsic" (i.e., apoptosome) and the "extrinsic" (i.e., death receptor and perforin/granzyme) pathways (Stennicke and Salvesen, 2000). Current evidence suggests the following model for activation of the intrinsic pathway of apoptosis. A death signal induces the release of mitochondrial proteins, such as cytochrome c (cyt c; Liu et al., 1996) and Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), through an unknown mechanism that may involve a pore formed by proapoptotic BCL-2 family proteins and/or the permeability transition pore. Once released, Smac/Diablo releases inhibitor-of-apoptosis protein–mediated (IAP-mediated) inhibition of procaspases (presumably at the level of the apoptosome), while cyt c forms a complex with Apaf-1 and procaspase-9, which in the presence of ATP or dATP becomes activated, resulting in further caspase activation, cleavage of cellular substrates, and cell death (Liu et al., 1996; Li et al., 1997; Zou et al., 1997).

In the nervous system, PCD occurs during both developmental and pathological processes. During development, 20%–80% of all neurons produced during embryogenesis die before reaching adulthood (Oppenheim, 1991), ostensibly to match the number of innervating neurons with the size of the target tissue. One of the most extensively studied models of neuronal PCD is nerve growth factor (NGF) deprivation in neonatal sympathetic neurons. This death, which requires de novo protein synthesis (Martin et al., 1988), BAX expression (Deckwerth et al., 1996), and caspase activation (Deshmukh et al., 1996; Troy et al., 1996; McCarthy et al., 1997), is blocked by neuroprotective agents, such as KCl and cAMP (Rydel and Greene, 1988; Koike et al., 1989; Edwards et al., 1991; Deckwerth and Johnson, 1993).

Trophic factor withdrawal in neonatal sympathetic neurons induces two parallel processes: a protein synthesis–dependent, caspase-independent translocation of BAX from the cytosol to mitochondria, followed by mitochondrial-membrane integration and loss of cyt c (Putcha et al., 1999, 2000); and the development of competence to die, which requires neither macromolecular synthesis nor BAX expression (Deshmukh and Johnson, 1998). Activation of both pathways is required for caspase activation and apoptosis in immature sympathetic neurons.

Over a decade ago, we reported that NGF deprivation–induced apoptosis in neonatal sympathetic neurons requires macromolecular synthesis, since multiple RNA *and* protein synthesis inhibitors prevent cell death in this paradigm (Martin et al., 1988). Based on these observations, we hypothesized that, true to its name, developmental PCD is a differentiation "program" like any other, requiring selective new gene expression designed to direct a cell to its physiologically appropriate fate—in this case, death. We also postulated the existence of certain genes, called "thanatins," that should have two properties: first, they should be induced during neuronal PCD; second, their principal, or sole, function should be to mediate apoptosis. More recent findings indicate that these thanatins effect cell death by regulating BAX-

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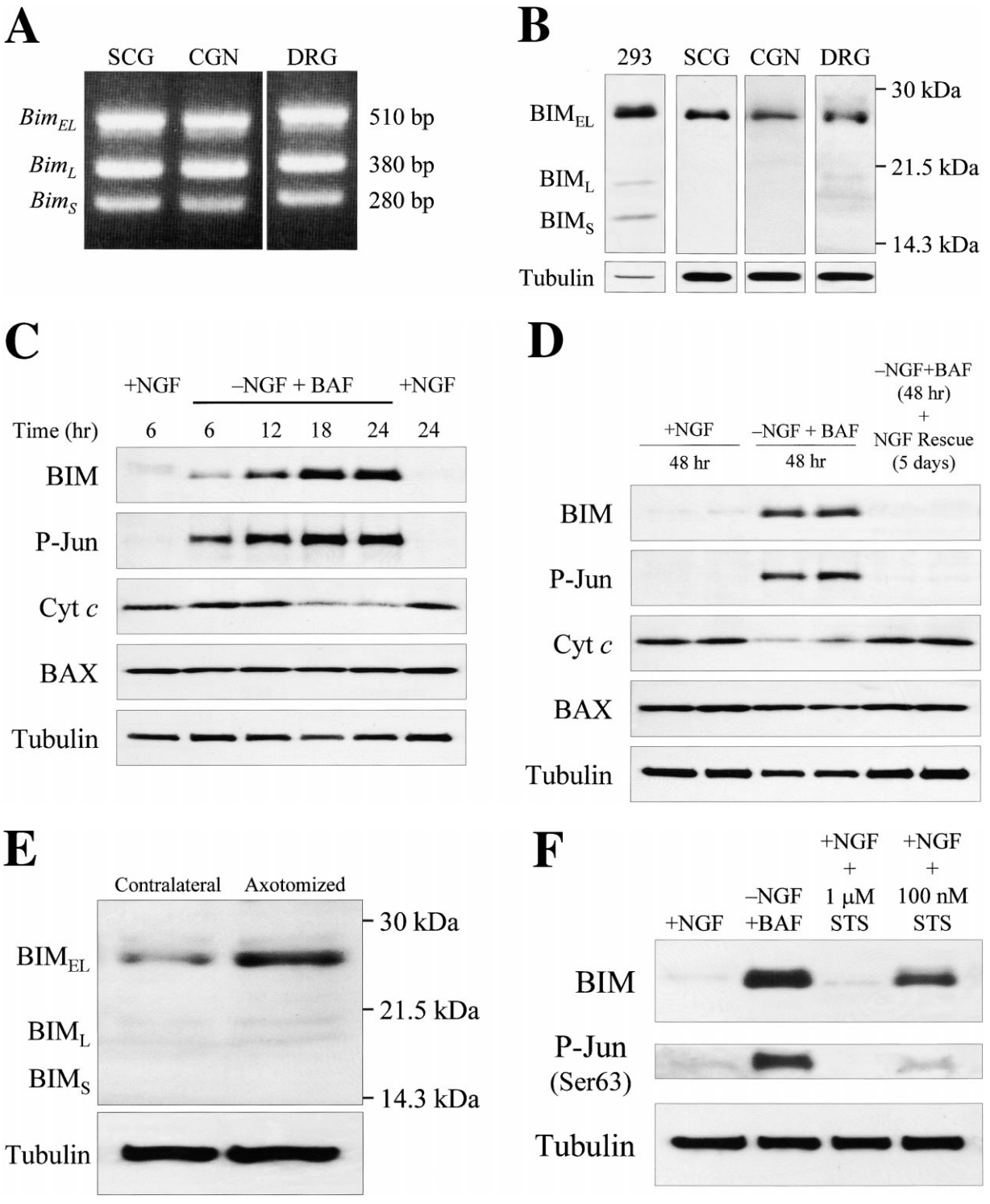

Figure 1. BIM_{EL} Is Induced during Neuronal Apoptosis Independent of Caspase Activation

(A) RT–PCR analysis of NGF-maintained DIV7 sympathetic neurons, K^+-maintained DIV7 CGNs, and P1 DRG neurons shows that these neurons express all three splice variants of *Bim*: Bim_S (280 bp), Bim_L (380 bp), and Bim_{EL} (~510 bp).

(B) Western analysis of DIV7 SCG, DIV7 CG, and P1 DRG neurons indicates that these neurons express primarily BIM_{EL}. Similarly, human embryonic kidney (293) cells express primarily BIM_{EL} but also show detectable levels of BIM_L and BIM_S.

(C) DIV5 sympathetic neurons were maintained in NGF or deprived of NGF in the presence of 50 μM BAF for the indicated times and then lysed. Expression of proteins was analyzed by immunoblotting. Tubulin immunoblotting confirmed equal protein loading.

(D) DIV5 SCG neurons were maintained in NGF or deprived of NGF in the presence of 50 μM BAF for 48 hr and harvested. In parallel, cultures of NGF-deprived, BAF-treated neurons were rescued at 48 hr by readdition of NGF; after 5 more days in the presence of NGF, these cells were also harvested. All extracts were then examined by Western blotting.

dependent cyt c release. However, the identities of these gene products are unknown.

Here, we report that NGF deprivation in neonatal sympathetic neurons rapidly induced expression of the extra-long splice variant of BIM, a member of the BH3-only proapoptotic subfamily of the BCL-2 family of proteins. Analysis of *Bim*-deficient mice underscores the importance of BIM induction in neuronal apoptosis. In both model systems examined—NGF deprivation in sympathetic neurons and K^+ withdrawal in cerebellar granule neurons (CGNs)—*Bim* deletion conferred protection against cyt c release and neuronal apoptosis. We propose that BIM and other BH3-only proteins are induced during neuronal apoptosis, serving partially redundant functions by which extracellular death signals such as trophic factor deprivation are transduced from the plasma membrane to mitochondria, culminating in BAX-mediated cyt c release, caspase activation, and cell death.

Results

The Proapoptotic BH3-Only Protein BIM_{EL} Is Induced during Neuronal Apoptosis

Apoptosis induced by trophic factor withdrawal in sympathetic neurons requires macromolecular synthesis (Martin et al., 1988). In nematodes, EGL-1 is induced in cells destined to die (Conradt and Horvitz, 1998; Chen et al., 2000); similarly, in neonatal rat sympathetic neurons, HRK/DP5 is induced during NGF deprivation (Imaizumi et al., 1997). Both EGL-1 and HRK/DP5 belong to the BH3-only proapoptotic subfamily of the BCL-2 family of proteins. Therefore, we hypothesized that members of this BH3-only subfamily are induced during sympathetic neuronal apoptosis and are critical for its execution. To test this hypothesis, we first examined the expression of various BH3-only proteins in two well-characterized models of neuronal apoptosis: NGF deprivation in superior cervical ganglion (SCG) neurons and K^+ withdrawal in CGNs.

Analysis of mRNA expression by RT–PCR demonstrated expression of all three splice variants of *Bim* (Bcl-2-interacting mediator of cell death; O'Connor et al., 1998) in the three neuronal subpopulations examined: NGF-maintained DIV7 SCG neurons, K^+-maintained DIV7 CGNs, and P1 dorsal root ganglion (DRG) neurons (Figure 1A). However, analysis of protein expression indicated that all three neuronal populations expressed primarily, if not exclusively, BIM_{EL} (Figure 1B), albeit at low levels, consistent with a recent report that BIM_L and especially BIM_{EL} may be the physiologically more relevant isoforms in neurons and that both are normally expressed at very low levels (O'Reilly et al., 2000).

We then examined whether BIM expression changed during trophic factor withdrawal in sympathetic neurons. NGF deprivation significantly induced the expression of both BIM_{EL} mRNA (data not shown) and protein (Figure 1C), starting at ~6–12 hr and peaking by ~18–24 hr. Similar induction was also seen with a different antibody (14A8; O'Reilly et al., 1998) to BIM (data not shown). Although Bim_L mRNA was also induced with a slightly slower time course than that for Bim_{EL}, we detected little, if any, induction of BIM_L protein (data not shown). Taken together with the results shown in Figures 1A and 1B, these observations may indicate a role for posttranscriptional regulation of BIM expression.

BIM_{EL} induction was observed in the presence or absence of the caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF); in fact, BAF did not alter either the time course or extent of BIM_{EL} induction, nor did BAF alone cause BIM_{EL} upregulation in NGF-maintained neurons (data not shown). Since upregulation of BIM_{EL} did not require caspase activation, these observations indicate that this induction event occurs upstream of (or in parallel with) the caspase checkpoint in sympathetic neurons.

In contrast to BIM_{EL}, which was significantly induced by NGF deprivation, expression of BAX was unchanged 48–72 hr after trophic factor withdrawal (Figures 1C, 1D, and 2C and data not shown), consistent with posttranslational regulation of the proapoptotic activity of BAX in these cells (Putcha et al., 1999, 2000). Moreover, BIM_{EL} induction preceded the loss of mitochondrial cyt c, which starts ~18 hr after trophic factor withdrawal and is complete by ~36 hr in neonatal rat sympathetic neurons (Figure 1C; Tsui-Pierchala et al., 2000).

Recent findings indicate that readdition of NGF can in the short term arrest and in the long term reverse the translocation of BAX to mitochondria and the loss of mitochondrial cyt c in NGF-deprived, BAF-treated SCG neurons (Deshmukh and Johnson, 1998; Martinou et al., 1999; Putcha et al., 1999). However, caspase inhibition only extends commitment to death for a finite period, from the point of cyt c release to the later point of mitochondrial depolarization (Deshmukh and Johnson, 2000a). Therefore, we also examined whether readdition of NGF within this "window of opportunity" could reverse upregulation of BIM_{EL} caused by trophic factor withdrawal. Figure 1D shows that readdition of NGF for 5 days after 48 hr of trophic factor deprivation can reverse c-Jun phosphorylation, BIM_{EL} induction, and loss of mitochondrial cyt c.

BIM Induction Is a Hallmark of Neuronal, but Not Nonneuronal, Apoptosis

To determine whether upregulation of BIM is a general characteristic of neuronal PCD, we examined BIM expression in two other well-characterized models of neuronal apoptosis: neonatal axotomy (Figure 1E) and K^+ deprivation in P7 mouse CGNs (Figure 4B). Like NGF deprivation in SCG neurons, apoptotic cell death in both of these paradigms requires de novo protein synthesis (Oppenheim et al., 1990; Miller and Johnson, 1996) and BAX expression (Deckwerth et al., 1996; Miller et al., 1997a). Accordingly, 24 hr after sciatic nerve axotomy

(E) Sciatic nerve axotomy was performed on neonatal rats. After 24 hr, the L4 and L5 DRGs from the lesioned and unlesioned sides were dissected, homogenized in lysis buffer, and analyzed by Western blotting.

(F) Sympathetic neurons (DIV5) were maintained in NGF, deprived of NGF in the presence of 50 μM BAF, or maintained in NGF in the presence of 1 μM or 100 nM STS. After 24 hr, extracts were prepared and examined by immunoblotting.

and 6–24 hr after K^+ withdrawal, BIM_{EL} was induced in L4 and L5 DRG neurons and CGNs, respectively. Thus, disparate apoptotic signals induced BIM_{EL} in neurons. In contrast, death-inducing stimuli such as dexamethasone, staurosporine (STS), and γ-irradiation do not induce expression of any BIM isoform in nonneuronal cells (O'Connor et al., 1998; data not shown). These observations suggest that induction of BIM_{EL} may represent a hallmark of neuronal, but not nonneuronal, apoptosis.

To evaluate whether upregulation of BIM expression characterized apoptotic, but not nonapoptotic, stimuli, we examined STS-induced cell death in sympathetic neurons. Low (100 nM) and high (1 μM) doses of STS induce death by different mechanisms in SCG neurons: whereas cell death caused by 100 nM STS resembles NGF deprivation–induced apoptosis in many respects (e.g., cyt c release, caspase activation, and TUNEL positivity), death caused by 1 μM STS does not, indicating activation of a nonapoptotic cell death pathway (Deshmukh and Johnson, 2000b). As shown in Figure 1F, treatment of sympathetic neurons with 100 nM, but not 1 μM, STS for 24 hr induced BIM_{EL} expression. These observations suggest that induction of BIM_{EL} may characterize apoptotic, but not nonapoptotic, neuronal cell death.

Neuroprotective Agents Prevent BIM Induction

Inhibitors of macromolecular synthesis, such as cycloheximide (CHX) and actinomycin D (ActD), prevent NGF deprivation–induced apoptosis in SCG neurons (Martin et al., 1988), aborting the cell death pathway upstream of BAX translocation, cyt c release, and caspase activation (Deshmukh and Johnson, 1998; Neame et al., 1998; Putcha et al., 1999). To determine whether de novo RNA and protein syntheses were required for BIM_{EL} induction, we examined the ability of ActD or CHX to block this event. Sympathetic neurons deprived of NGF for 24 hr in the presence of either ActD (0.1 μg/ml) or CHX (1 μg/ml) showed little, if any, upregulation of BIM_{EL} expression (Figures 2A and 2B).

Although NGF is the physiological survival factor for sympathetic neurons in vivo, several other agents can promote their survival in vitro. Of these, two robust neuroprotectants are the cell-permeable cAMP analog, CPT-cAMP, and depolarizing concentrations of KCl (Rydel and Greene, 1988; Koike et al., 1989; Edwards et al., 1991; Deckwerth and Johnson, 1993; Franklin et al., 1995). Both neuroprotectants abort the cell death pathway induced by trophic factor withdrawal upstream of BAX translocation, cyt c release, and caspase activation (Neame et al., 1998; Putcha et al., 1999). Therefore, we examined whether these agents prevent BIM_{EL} upregulation during sympathetic neuronal death. As shown in Figure 2C, even after 24 hr of NGF deprivation CPT-cAMP (400 μM) or KCl (40 mM) prevented BIM_{EL} induction. Thus, trophic factor withdrawal in SCG neurons significantly induced a macromolecular synthesis–dependent, caspase-independent induction of BIM_{EL} that is prevented by neuroprotectants, such as cAMP and KCl.

Neither JNK Activation nor PI3K Inhibition Is Required for BIM Induction

Multiple signaling pathways may modulate the NGF-dependent survival of sympathetic neurons, including

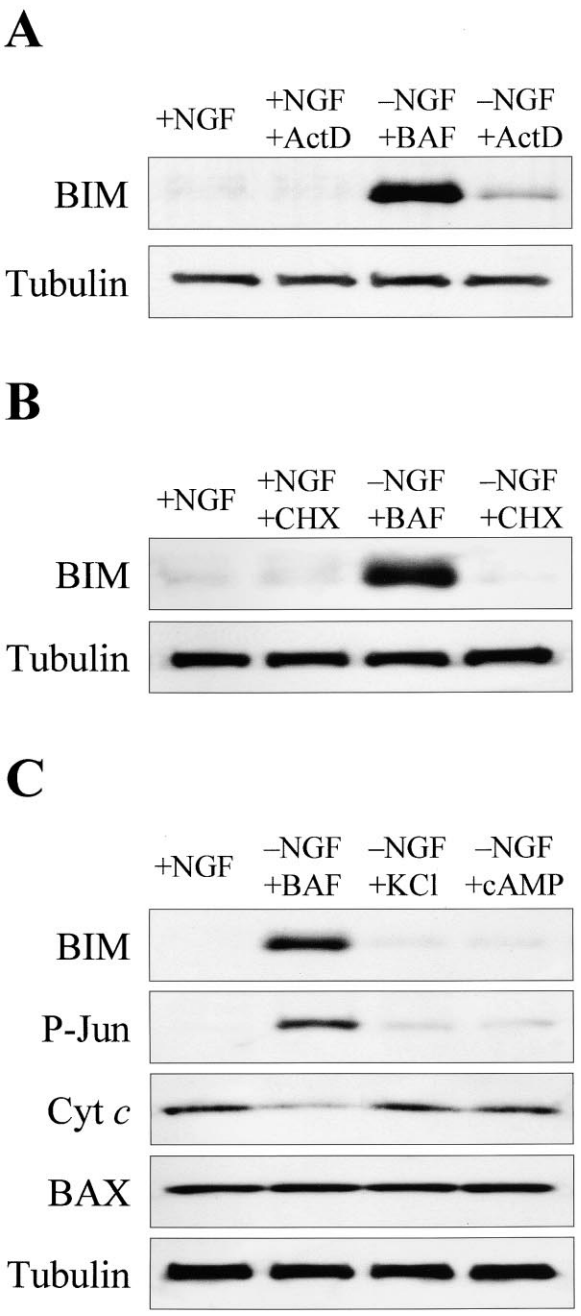

Figure 2. Inhibitors of Macromolecular Synthesis and Neuroprotectants Prevent NGF Deprivation–Induced BIM_{EL} Expression in Sympathetic Neurons

(A) DIV5 SCG neurons were maintained in NGF in the presence or absence of 0.1 μg/ml ActD or deprived of NGF in the presence of 50 μM BAF or 0.1 μg/ml ActD. After 24 hr, whole-cell extracts were prepared and examined by immunoblotting.

(B) Sympathetic neurons (DIV5) were maintained in NGF in the presence or absence of 1 μg/ml CHX or deprived of NGF in the presence of 50 μM BAF or 1 μg/ml CHX. After 24 hr, cells were lysed and evaluated by Western blotting.

(C) DIV5 SCG neurons were maintained in NGF or deprived of NGF in the presence of 50 μM BAF, 40 mM KCl, or 400 μM CPT-cAMP. After 24 hr, extracts were prepared and analyzed by immunoblotting.

extracellular-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), p38 mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K), and Cdc42/Rac. Although the physiological significance, functions, targets, and interactions of these pathways remain uncertain, two have recently received considerable attention: PI3K and JNK.

PI3K may promote survival primarily through activation of the serine/threonine kinase Akt/protein kinase B (PKB; Dudek et al., 1997), whose targets include BAD (Datta et al., 1997; del Peso et al., 1997), caspase-9 (Cardone et al., 1998), and Forkhead transcription factors (Biggs et al., 1999; Brunet et al., 1999). Although PI3K is necessary and sufficient for the K^+- and insulin-mediated survival of CGNs (D'Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997b), its precise function in SCG neurons, in which PI3K completely accounts for the trophic but not the survival-promoting actions of NGF (Tsui-Pierchala et al., 2000), is less clear (Philpott et al., 1997; Crowder and Freeman, 1998; Mazzoni et al., 1999; Vaillant et al., 1999). Variations in the subcellular localization of PI3K signal initiation and propagation may regulate these activities (Kuruvilla et al., 2000) but cannot completely explain discrepancies in the literature.

To determine whether PI3K activity regulates BIM, we treated NGF-maintained sympathetic neurons with 50 μM LY294002 and examined BIM_{EL} expression after 24 hr (Figure 3A). As shown previously (Tsui-Pierchala et al., 2000), this concentration of LY294002 completely inhibited PI3K-dependent Akt (S473 and T308) phosphorylation and induced S63 phosphorylation of c-Jun. However, LY294002 did not induce BIM_{EL} expression, even at earlier and later time points (data not shown). In contrast, NGF deprivation, which was associated with loss of P-Akt (S473), caused robust c-Jun (S63) phosphorylation and BIM_{EL} induction. Thus, in contrast to other genes induced by trophic factor withdrawal (Crowder and Freeman, 1998; Lipscomb et al., 1999), BIM expression was not significantly increased by LY294002 and, therefore, may help explain why PI3K signaling alone is not wholly responsible for the NGF-dependent survival of sympathetic neurons, at least in mass cultures.

In contrast to PI3K, which may contribute to NGF-mediated survival, activation of the JNK signaling pathway promotes sympathetic neuronal apoptosis. For example, JNK activity and c-Jun (S63) phosphorylation, which enhances c-Jun-mediated transactivation (Pulverer et al., 1991; Smeal et al., 1991), increase dramatically 6–12 hr after NGF deprivation (Figure 1C; Ham et al., 1995; Virdee et al., 1997; Eilers et al., 1998; Tsui-Pierchala et al., 2000). Moreover, inhibiting JNK activation (Maroney et al., 1999) or c-Jun function (Estus et al., 1994; Ham et al., 1995) attenuates apoptosis caused by trophic factor withdrawal in SCG neurons. The mechanism by which JNK and c-Jun promote neuronal apoptosis remains unclear; however, one hypothesis is that JNK activates c-Jun, which in turn is responsible for the transactivation of proapoptotic genes, such as BIM, that trigger BAX-dependent cyt c release. Consistent with this hypothesis, c-Jun phosphorylation accompanied BIM_{EL} induction in both SCG neurons and CGNs (Figures 1C and 4B, respectively).

Therefore, we examined whether activation of the JNK signaling pathway is required for BIM induction. First, we treated NGF-maintained and -deprived SCG neurons with CEP-1347 (KT7515) for 24 hr and then examined BIM expression by Western analysis. CEP-1347, which has been shown to prevent death caused by trophic factor withdrawal in both sympathetic (Maroney et al., 1999) and motor neurons (Maroney et al., 1998), is an indolocarbazole of the K252a family that does not inhibit JNKs directly but instead acts upstream of JNKs to block their activation and c-Jun phosphorylation. As shown in Figure 3B, CEP-1347 completely prevented c-Jun (S63) phosphorylation, but not BIM_{EL} induction, caused by NGF deprivation; CEP-1347 alone did not cause upregulation of BIM_{EL} in NGF-maintained cells (data not shown). Second, 100 nM STS, which functions as a broad-spectrum kinase inhibitor, induced BIM_{EL} expression but not c-Jun phosphorylation (Figure 1F); conversely, 50 μM LY294002 caused c-Jun phosphorylation but not BIM_{EL} induction (Figure 3A). Thus, activation of the JNK signaling pathway was not required for NGF deprivation–induced BIM_{EL} expression in sympathetic neurons.

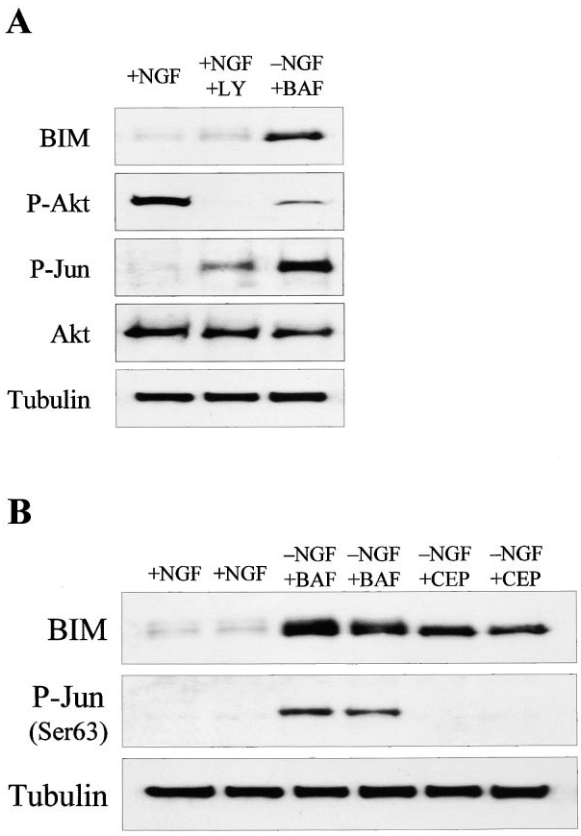

Figure 3. Neither PI3K Inhibition nor JNK Activation Is Required for BIM Induction

(A) Sympathetic neurons (DIV5) were maintained in NGF in the presence or absence of 50 μM LY294002, a reversible PI3K inhibitor, or deprived of NGF in the presence of 50 μM BAF. After 24 hr, cells were harvested and examined by immunoblotting.

(B) DIV5 SCG neurons were maintained in NGF or deprived of NGF in the presence of 50 μM BAF or 250 nM CEP-1347, an inhibitor of JNK activation. After 24 hr, whole-cell extracts were prepared and analyzed by Western blotting.

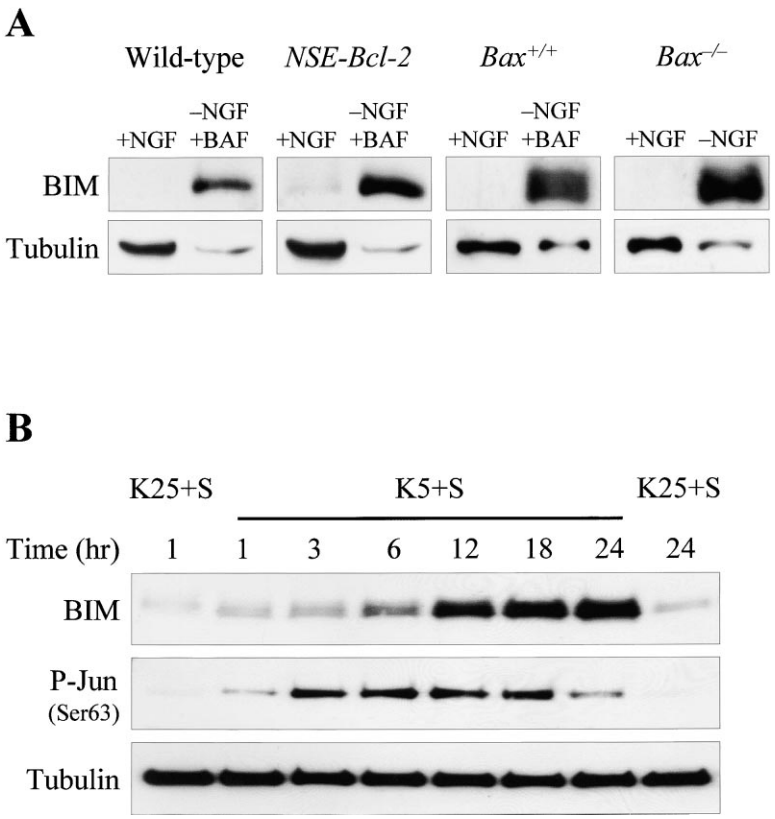

Figure 4. Induction of BIM_{EL} in Both Central and Peripheral Neurons Occurs Upstream of the BAX/BCL-2 Checkpoint

(A) Sympathetic neurons (DIV5) from wild-type, *NSE-Bcl-2* transgenic, $Bax^{+/+}$, or $Bax^{-/-}$ mice were maintained in NGF or deprived of NGF in the presence or absence of 50 μM BAF for 48 hr. Cells were then lysed and examined by immunoblotting.

(B) CGNs (DIV7) from $Bax^{-/-}$ mice were maintained in K25+S or in K5+S for various periods. At the indicated times, whole-cell extracts were prepared and analyzed by Western blotting.

BIM Induction Lies Upstream of the BAX/BCL-2 and Caspase Checkpoints

Since caspase inhibition did not affect BIM_{EL} induction, we evaluated whether BIM also functioned upstream of the BAX/BCL-2 checkpoint by examining BIM expression after NGF deprivation in sympathetic neurons from *NSE-Bcl-2* transgenic (Martinou et al., 1994) and $Bax^{-/-}$ (Knudson et al., 1995) mice. As shown in Figure 4A, trophic factor withdrawal caused induction of BIM_{EL} in both *Bcl-2*-overexpressing and *Bax*-deficient SCG neurons. Moreover, neither overexpression of *Bcl-2* nor deletion of *Bax* altered the time course or extent of BIM_{EL} induction (data not shown). Similarly, K^+ deprivation in CGNs from $Bax^{-/-}$ or *Bcl-2* transgenic mice also caused induction of BIM_{EL} (Figure 4B and data not shown, respectively). In sum, BIM induction occurred upstream of (or in parallel with) the BAX/BCL-2 and caspase checkpoints.

When Induced, BIM Resides in Mitochondria as an Integral Membrane Protein

The sequestration of cell death effectors, such as BAX and cyt c, within distinct subcellular compartments has recently emerged as a common theme in the regulation of apoptosis. Therefore, we examined the localization of endogenous BIM by using subcellular fractionation. As shown in Figure 5A, NGF-maintained sympathetic neurons expressed little if any BIM_{EL}; however, as seen previously in whole-cell lysates (Figures 1–4), NGF deprivation significantly induced BIM_{EL} expression. Moreover, when induced, BIM_{EL} was found exclusively in the heavy membrane fraction enriched in mitochondria. To determine whether BIM_{EL} associated with mitochondria is integrated into mitochondrial membranes, we performed alkali extraction of the mitochondrial fractions from NGF-maintained and -deprived sympathetic neurons. In this assay, mitochondrial integral membrane proteins are resistant to extraction by 0.2 M Na_2CO_3 yet sensitive to extraction by 1% SDS, whereas peripheral membrane proteins are extracted by both treatments. Using this assay, we previously demonstrated that, after translocation from the cytosol to mitochondria, BAX integrates into the mitochondrial outer membrane (Putcha et al., 2000). Similar analysis of BIM indicated that, when induced, BIM_{EL} was an integral membrane protein in mitochondria (Figure 5B). These observations suggest that BIM_{EL} was unlikely to be responsible for the translocation of BAX from the cytosol to mitochondria, but instead contributed to the multimerization and integration of BAX into the mitochondrial outer membrane and/or BAX-mediated cyt c release (see Discussion).

Bim Deletion Confers Transient Protection against Apoptosis in Central and Peripheral Neuronal Populations

The experiments described above indicate that apoptotic stimuli significantly induced expression of BIM_{EL}, a BH3-only protein residing as an integral membrane protein in mitochondria and functioning upstream of the BAX/BCL-2 checkpoint. These observations suggest that BIM induction may be critical for neuronal apoptosis. To test this hypothesis directly, we examined both developmental and induced neuronal apoptosis in *Bim*-deficient mice, which exhibit altered leukocyte

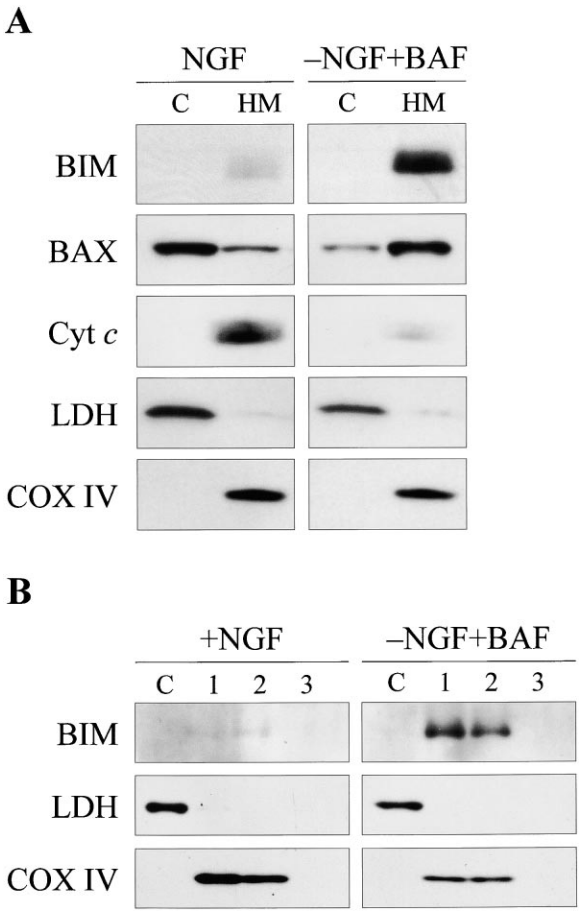

Figure 5. When Induced, BIM Resides in Mitochondria as an Integral Membrane Protein

(A) Sympathetic neurons (DIV5) were maintained in NGF or deprived of NGF in the presence of 50 μM BAF for 30 hr, and the subcellular localization of BIM, BAX, and cyt c was examined by fractionation. Lactate dehydrogenase and cytochrome oxidase subunit IV served as markers for the purity of cytosolic and heavy membrane fractions, respectively, and as markers for equal protein loading. Markers for the endoplasmic reticulum, Golgi, and plasma membrane labeled only the microsomal fraction (not depicted here). Please note that the BAX and cyt c blots have been reported previously in Putcha et al. (2000) and are included only for comparison.

(B) DIV5 SCG neurons were maintained in NGF or deprived of NGF in the presence of 50 μM BAF for 30 hr, and the subcellular localization of BIM was examined by fractionation with alkali extraction by using 0.2 M Na_2CO_3 (pH 11.5). SDS (1% w/v) served as a positive control for the extraction of integral membrane proteins.

Abbreviations: C, cytosol; HM, heavy membrane; LDH, lactate dehydrogenase; COX IV, cytochrome oxidase subunit IV; 1, isotonic buffer; 2, 0.2 M Na_2CO_3; 3, 1% SDS).

homeostasis, selective apoptotic deficits in lymphocytes, shortened lifespans, and significant embryonic lethality (65% by E9.5; Bouillet et al., 1999; O'Reilly et al., 2000).

We determined whether sympathetic neurons and CGNs from $Bim^{-/-}$ animals were protected from apoptosis induced by trophic factor withdrawal and K^+ deprivation, respectively. As shown in Figure 6A, *Bim* deletion protected CGNs from K^+ withdrawal, but only transiently, extending the 50% viability point from ~12 hr to ~24 hr. $Bim^{-/-}$ sympathetic neurons showed a similar delay of ~12–14 hr in the time when they became committed to die, defined as the time when 50% of the cells cannot be rescued by readdition of NGF (data not shown). Furthermore, both SCG and CG neurons displayed a modest, but reproducible, gene dosage effect for *Bim*, suggesting that BIM may function at a rate-limiting step during neuronal apoptosis. However, in neither sympathetic neurons nor CGNs did *Bim* deletion impart complete protection as does *Bax* deficiency (Deckwerth et al., 1996; Miller et al., 1997a), suggesting that functional redundancy occurs among BH3-only proteins.

As indicated above, trophic factor withdrawal initiates a sequence of molecular and biochemical events culminating in apoptotic cell death in sympathetic neurons. NGF-deprived sympathetic neurons arrested at the BAX/BCL-2 checkpoint (e.g., by *Bax* deletion or *Bcl-2* overexpression) still undergo early signaling events (e.g., c-Jun phosphorylation) but not late ones (e.g., cyt c release), whereas cells treated with caspase inhibitors undergo both early and late events (Deckwerth et al., 1998; Deshmukh and Johnson, 1998; Neame et al., 1998; Martinou et al., 1999; Putcha et al., 1999, 2000). Therefore, we examined where in the signaling cascade initiated by trophic factor withdrawal *Bim* deletion inhibits cell death. We deprived $Bim^{+/+}$, $Bim^{+/-}$, and $Bim^{-/-}$ sympathetic neurons of NGF for various periods, immunostained them for P-Jun(S63) or cyt c, and counted the number of cells with intense, nuclear P-Jun or diffuse, cytosolic cyt c staining. As seen in Figures 6B and 6C, respectively, *Bim* deletion did not alter the kinetics of c-Jun (S63) phosphorylation but clearly delayed the time course of cyt c release. Moreover, the time course of cyt c release was indistinguishable from that for commitment to die, indicating once again that, in the absence of caspase inhibition, the loss of mitochondrial cyt c irreversibly commits sympathetic neurons to die after trophic factor withdrawal (Deshmukh and Johnson, 1998; Putcha et al., 1999; Deshmukh and Johnson, 2000a; data not shown). However, *Bim* deletion did not completely prevent cyt c release, as does *Bax* deficiency (Figure 6C), lending further support to the hypothesis that BH3-only proteins are functionally redundant.

Bim Deletion Attenuates Programmed Cell Death during Neuronal Development

We next examined whether *Bim* deficiency, which conferred neuroprotection in vitro, also inhibited naturally occurring neuronal PCD in vivo. Since targeted deletion of *Bim*, unlike *Bax*, conferred only transient protection against neuronal apoptosis in vitro (Figure 6), we hypothesized that any delay in neuronal PCD in vivo would be transitory and, therefore, directed our preliminary analysis to DRG neurons at E14.5, during the peak of naturally occurring cell death (White et al., 1998). We sectioned E14.5 $Bim^{+/+}$ and $Bim^{-/-}$ littermates, examined DNA fragmentation by using a TUNEL assay, and counted the number of TUNEL-positive neurons. As shown in Figure 7, *Bim* deletion reduced the number of TUNEL-positive thoracic and lumbar DRG neurons by ~50% ($p < 0.001$). However, no apparent differences were observed in TUNEL staining for several nonneu-

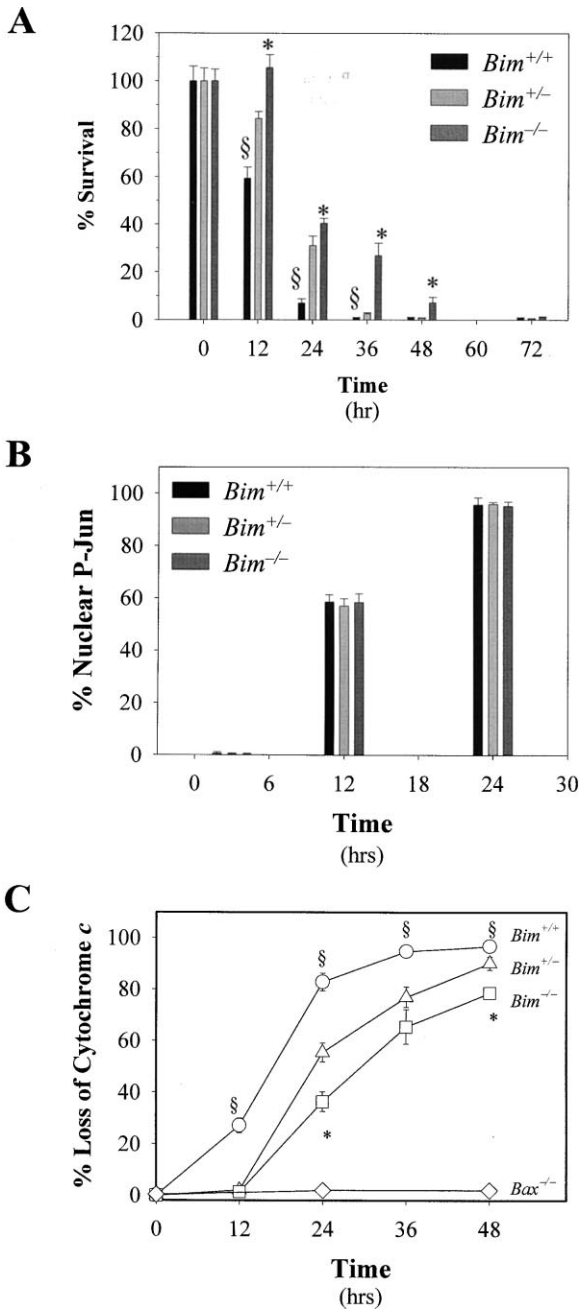

Figure 6. *Bim* Deletion Delays but Does Not Prevent Cyt c Release and Apoptosis in Central and Peripheral Neurons

(A) CGNs (DIV7) from $Bim^{+/+}$, $Bim^{+/-}$, or $Bim^{-/-}$ mice were maintained in K25+S or K5+S for various periods. At the indicated times, cells were stained with the vital dye calcein AM and photographed. A naïve observer then counted the number of calcein AM–stained neurons in the photomicrographs. Symbols denote statistically significant differences (a section mark indicates that $Bim^{+/+}$ differed from $Bim^{+/-}$ and $Bim^{-/-}$, $p \leq 0.003$; an asterisk indicates that $Bim^{+/-}$ differed from $Bim^{-/-}$, $p < 0.05$; mean ± SEM; n = 8–13).

(B and C) Sympathetic neurons (DIV5) from $Bim^{+/+}$, $Bim^{+/-}$, or $Bim^{-/-}$ mice were maintained in NGF or deprived of NGF in the presence of 50 μM BAF for various periods. At the indicated times, cells were fixed and immunostained for P-Jun (Ser63) or cyt c. Then, the number of cells exhibiting intense, nuclear P-Jun (B) or diffuse cytosolic cyt c (C) was determined by a naïve observer. At all time points, NGF-maintained neurons showed neither nuclear P-Jun nor cytosolic cyt c. Symbols denote statistically significant differences (a section mark indicates that $Bim^{+/+}$ differed from $Bim^{+/-}$ and $Bim^{-/-}$, $p \leq 0.041$; an asterisk indicates that $Bim^{+/-}$ differed from $Bim^{-/-}$, $p \leq 0.005$; mean ± SEM; n = 3–7 [B], n = 3–12 [C]).

ronal structures, such as the interdigital web (Figures 7C and 7D). Thus, *Bim* deficiency delayed but did not prevent apoptosis both in vitro and presumably in vivo in both central and peripheral neurons, but not in certain nonneuronal cell types.

Discussion

NGF deprivation in SCG neurons rapidly and significantly induced expression of BIM_{EL} in *Bcl2*-overexpressing, *Bax*-deficient, or caspase inhibitor–treated sympathetic neurons, indicating that this event lies upstream of the BAX/BCL-2 and caspase checkpoints. BIM_{EL} was also induced during cell death caused by other apoptotic, but not nonapoptotic, stimuli (e.g., staurosporine) and in other neuronal subpopulations (e.g., DRG neurons), suggesting that the induction of BIM may constitute a hallmark of neuronal apoptosis. However, this event appears to be unique to neurons, since BIM is *not* induced in several nonneuronal models of cell death. Subcellular localization studies indicated that, when induced, BIM was an integral membrane protein in mitochondria, suggesting that BIM probably did not mediate the cytosol-to-mitochondria translocation of BAX but instead contributed to BAX-dependent cyt c release. Finally, *Bim* deficiency delayed, but did not prevent, developmental and induced PCD in both central and peripheral neurons, suggesting that functional redundancy exists among BH3-only proteins.

The Proapoptotic BH3-Only Protein BIM Is Induced during Sympathetic Neuronal Apoptosis

Since the initial report demonstrating that NGF deprivation–induced apoptosis in sympathetic neurons requires macromolecular synthesis (Martin et al., 1988), the search for those genes, dubbed "thanatins" (Johnson et al., 1989), upregulated during apoptosis—the raison d'être of which is to mediate cell death—has been an area of active investigation. Until recently, genes reported to be induced during neuronal death fell primarily into three distinct categories: transcription factors of the AP-1 subfamily (e.g., c-*jun* and c-*fos*), cell cycle regulators (e.g., c-*myb* and *cyclinD1*), and extracellular matrix proteases (e.g., *transin* and *collagenase*; Estus et al., 1994; Freeman et al., 1994); however, no component of the core apoptotic machinery is induced by trophic factor withdrawal in these neurons.

More recently, Imaizumi et al. (1997) and Inohara et al. (1997) reported the cloning of HRK/DP5, a proapoptotic member of the BCL-2 protein family that is induced by NGF deprivation in sympathetic neurons (Imaizumi et al., 1997). Overexpression of HRK/DP5, which contains only BH3 and transmembrane domains, causes cell death in both neuronal and nonneuronal cells that can be attenuated by co-overexpression of BCL-2 or BCL-X_L, possibly through direct BH3 domain–mediated interactions. Furthermore, although the subcellular distribu-

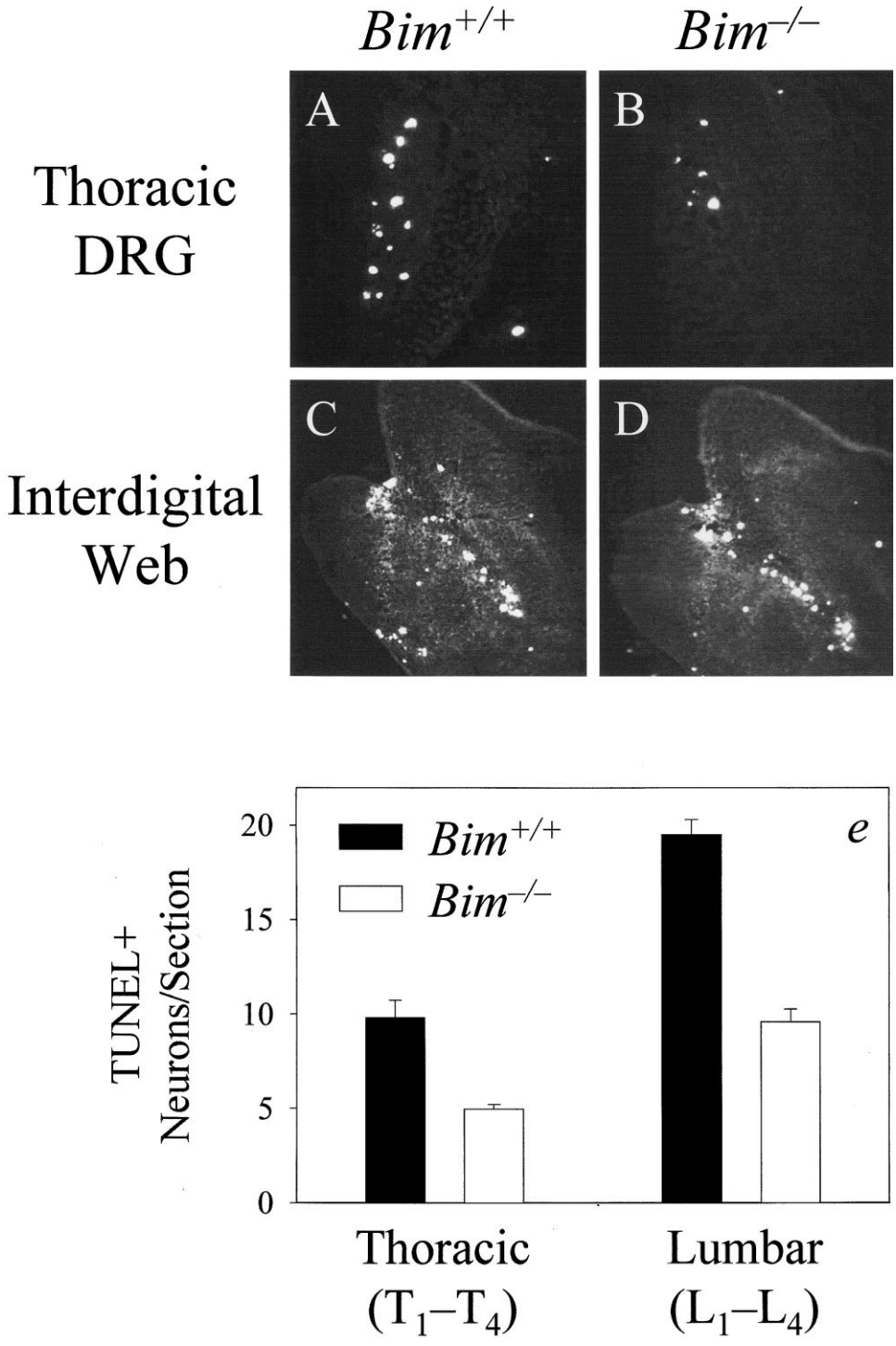

Figure 7. *Bim* Deficiency Attenuates Programmed Cell Death In Vivo during Neuronal but Not Nonneuronal Development

Naturally occurring PCD in thoracic (T1–T4) and lumbar (L1–L4) DRG neurons and in certain nonneuronal structures (e.g., the interdigital web of the forefoot) was examined in sections from E14.5 $Bim^{+/+}$ and $Bim^{-/-}$ embryos by TUNEL staining. Then, the number of TUNEL-positive DRG neurons per section was counted by a naïve observer (mean ± SEM, n = 3 embryos, $p < 0.001$).

tion of endogenous HRK/DP5 is unknown, overexpression of epitope-tagged HRK/DP5 indicates localization to intracytoplasmic membranes. Thus, HRK/DP5 may be a bona fide thanatin; however, the physiological function of endogenous HRK/DP5 remains unclear. In this paper, we have identified and functionally characterized another molecule that satisfies the criteria for a thanatin: BIM, which like HRK/DP5 is a member of the emerging BH3-only proapoptotic subfamily of the BCL-2 family of proteins.

Neonatal SCG, DRG, and CG neurons expressed mRNA for all three splice variants of *Bim*, yet expressed primarily BIM_{EL} protein (Figures 1A and 1B; O'Reilly et al., 2000). Like HRK/DP5 (Imaizumi et al., 1997; Inohara et al., 1997), BIM normally appears to be expressed at relatively low levels in neurons both in vivo (O'Reilly et al., 2000) and in vitro (Figure 1B and data not shown). However, apoptotic insults significantly induced BIM_{EL} expression. For example, disparate death signals such as NGF withdrawal (Figures 1–5), sciatic nerve axotomy (Figure 1E), and K^+ deprivation (Figure 4B) induced BIM_{EL} in SCG, DRG, and CG neurons, respectively. In contrast, other intrinsic pathway signals such as dexamethasone, STS, and γ-irradiation do not induce expression of any BIM isoform in nonneuronal cells (O'Connor et al., 1998; data not shown). Moreover, apoptotic insults, such as 100 nM STS, caused BIM_{EL} induction in neurons, whereas nonapoptotic stimuli, such as 1 μM STS, did not (Figure 1F). Taken together, these observations suggest that induction of BIM_{EL}, and perhaps of HRK/DP5 (Imaizumi et al., 1997), constitutes a hallmark of neuronal, but not nonneuronal, apoptosis.

To our knowledge, BIM represents the first gene product induced in a macromolecular synthesis–dependent model of apoptotic cell death whose genetic deletion confers protection in that same model, thereby providing formal proof that PCD in these paradigms is an active cell-fate decision like any other differentiation pathway requiring new gene expression.

BIM Is an Integral Membrane Protein in Mitochondria that Regulates BAX-Dependent Cyt c Release

The time course of BIM_{EL} induction was consistent with BIM causing BAX translocation and the subsequent BAX-mediated loss of mitochondrial cyt c (Figure 1D). However, subcellular localization studies indicated that, when induced, BIM_{EL} was an integral membrane protein in mitochondria, suggesting that BIM_{EL} probably did not trigger the translocation of BAX to mitochondria but instead contributed to the multimerization and integration of BAX into the mitochondrial outer membrane and/or BAX-dependent cyt c release. Since technical limitations hindered our efforts to examine directly whether *Bim* deletion affected the translocation, multimerization, or integration of BAX, exactly how BIM functions is unclear. We propose two possibilities that need not be mutually exclusive. First, BIM and presumably HRK/DP5 may directly interact with and inactivate antiapoptotic BCL-2 family members such as BCL-2 and BCL-X_L at the mitochondrial outer membrane (Puthalakath et al., 1999; Strasser et al., 2000), thereby freeing BAX to multimerize and integrate into the outer membrane, forming pores that release cyt c. Alternatively, BH3-only proteins such as BIM and HRK/DP5 may themselves constitute members of a multiprotein pore complex in which BAX (at least in many neurons) is an obligate component and which is responsible for the release of intermembrane-space proteins such as cyt c. In this model, BIM and/or HRK/DP5 presumably would *indirectly* modulate the activity of a multimeric BAX pore, since neither interacts directly with BAX in co-overexpression and yeast two-hybrid studies (Inohara et al., 1997; O'Connor et al., 1998).

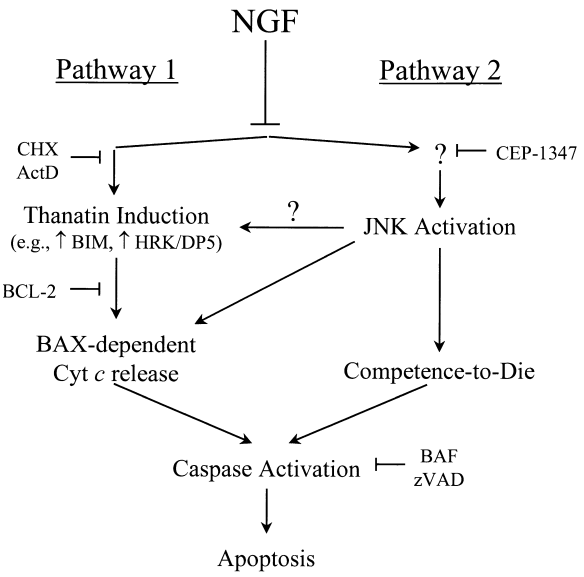

Figure 8. NGF Deprivation Causes the Activation of Parallel Pathways Culminating in Caspase Activation and Apoptosis

Pathway 1 involves the induction of proapoptotic genes ("thanatins"), such as BIM and HRK/DP5, which are responsible for the translocation and mitochondrial-membrane integration of BAX and/or BAX-dependent release of apoptogenic intermembrane-space proteins such as cyt c. (Although pore formation appears to require translocation, multimerization, and integration of BAX, whether multimerization precedes or follows translocation is unclear, and the identities of the gene products responsible for triggering BAX translocation remain unknown.) Pathway 2, which involves activation of the JNK pathway, is required for the development of competence to die and may contribute to, although it is not required for, thanatin induction (Harris et al., submitted). How these parallel pathways converge to mediate cyt c release is unknown.

BIM Induction and JNK Activation Represent Parallel Signaling Pathways Required for Neuronal Apoptosis

Of the numerous signal transduction cascades that may mediate survival signaling, two that have received considerable attention are the PI3K and JNK pathways. Activation of the former promotes survival (Datta et al., 1999), whereas signaling by the latter facilitates cell death (Davis, 2000). In SCG neurons, the necessity and sufficiency of either pathway for life or death, respectively, are controversial. Our observations indicate that neither JNK activation nor PI3K inhibition is required for NGF deprivation–induced BIM expression (Figures 1 and 3); in contrast, inhibition of the MEK/MAPK pathway causes BIM induction in NGF-maintained SCGs (G. V. P. and E. M. J., unpublished data). Therefore, our findings suggest that sympathetic neuronal apoptosis caused by trophic factor withdrawal requires the activation of parallel pathways, each of which (at physiological levels of expression) is necessary but not sufficient for complete and efficient cell death (Figure 8). Specifically, NGF deprivation induces the expression of thanatins, such as BH3-only proteins like BIM and HRK/DP5 (pathway 1), which in turn mediate the translocation of BAX from the cytosol to mitochondria and/or BAX-dependent cyt c release. In parallel, pathway 2 culminates in activation of the JNK signaling pathway, which in turn can regulate

not only cyt c release but also the development of competence to die (C. A. Harris, M. Deshmukh, A. Maroney, and E. M. J., submitted). Accordingly, inhibitors of macromolecular synthesis abort only pathway 1, whereas upstream inhibitors of JNK, such as CEP-1347, primarily block pathway 2. These findings suggest that BIM induction and JNK activation represent parallel pathways that are critical for cyt c release and sympathetic neuronal apoptosis under physiological conditions. Precisely how pathways 1 and 2 converge to mediate cyt c release is unclear.

Bim Deletion Delays, but Does Not Prevent, Induced and Developmental Cell Death in the Nervous System

Analysis of $Bim^{-/-}$ mice defines the importance of BIM function in neuronal apoptosis. In both model systems examined—NGF deprivation in sympathetic neurons and K^+ deprivation in CGNs—*Bim* deletion conferred protection against the loss of mitochondrial cyt c and neuronal apoptosis. However, the protection conferred by *Bim* deficiency was transient: $Bim^{-/-}$ sympathetic neurons and CGNs ultimately released cyt c (Figure 6C) and underwent an apoptotic cell death (Figure 6A and data not shown). Furthermore, developmental PCD in some neuronal (e.g., DRG neurons), but not some nonneuronal (e.g., interdigital web), cell types was attenuated in $Bim^{-/-}$ mice in vivo (Figure 7 and data not shown). However, this phenotype, like its in vitro counterpart, was not as robust as that seen in $Bax^{-/-}$ mice (White et al., 1998), suggesting some functional redundancy among BH3-only proteins upstream of BAX in neurons. This redundancy may not, however, extend to all BH3-only proteins. For example, targeted deletion of another BH3-only subfamily member, BAD, does not prevent cell death in E12 telencephalic neurons (Shindler et al., 1998). Moreover, *Bad* deficiency neither prevented nor altered the kinetics of cyt c release or commitment to die in NGF-deprived sympathetic neurons (G. V. P., S. J. Korsmeyer, and E. M. J., unpublished data).

In sum, our observations suggest that BIM served a partially redundant function upstream of the BAX/BCL-2 and caspase checkpoints. The reported or predicted similarities in the structure, function, subcellular localization, and time course of induction for endogenous BIM and HRK/DP5 are striking, suggesting that BIM and HRK/DP5 are two critical BH3-only proteins, upon which intrinsic pathway apoptotic signals converge in postmitotic neurons, ultimately leading to BAX-dependent cyt c release and cell death. The relative contribution of BIM or HRK/DP5 to neuronal apoptosis will likely vary according to the particular cell type and death stimulus. Based on our observations, we predict that neurons from *Hrk*/*Dp5*-deficient mice, if viable, will exhibit a partially protective phenotype like that seen with $Bim^{-/-}$ cells. Cyt c release and caspase activation will be delayed, but not prevented, as in $Bim^{-/-}$ neurons. Moreover, reproduction of the neuronal phenotype seen in $Bax^{-/-}$ mice, in which cyt c release and apoptotic cell death are completely prevented in many neurons, may require inactivation of at least both BIM and HRK/DP5.

More generally, death signals proceeding through the intrinsic pathway share certain common themes that have been conserved through evolution, at least from nematodes to mammals. First, death signals converge at the "activation" (e.g., by gene induction in macromolecular synthesis–dependent paradigms) of BH3-only death ligands, such as EGL-1, BIM, or HRK/DP5. These proteins then modulate the activities of "canonical" BCL-2 family proteins, inhibiting the activity of antiapoptotic members (e.g., CED-9, BCL-2, or BCL-X_L) and/or promoting the activity of proapoptotic members (e.g., BAX or BAK), leading (at least in vertebrates) to the release of apoptogenic proteins, such as cyt c and Smac/Diablo, which mediate the formation and activation, respectively, of an apoptosome consisting of adaptors (e.g., CED-4 or Apaf-1) and procaspases. Eventually, caspase activation, substrate cleavage, and apoptosis ensue. Although the specific "actors" in each particular play may vary (according to cell type and death stimulus), the broad themes of this genre appear exquisitely consistent.

Experimental Procedures

Reagents

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Other reagents and their sources were: collagenase and trypsin (Worthington Biochemical, Freehold, NJ); the caspase inhibitor BAF (Enzyme Systems Products, Livermore, CA); LY294002 (Biomol, Plymouth Meeting, PA); and CEP-1347 (KT7515; Dr. Anna Maroney, Cephalon, West Chester, PA). Medium lacking NGF (AM0) consisted of Eagle's MEM with Earle's salts (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine, 20 μM uridine, and 3.3 μg/ml aphidicolin. AM50 medium consisted of AM0 medium plus 50 ng/ml mouse 2.5S NGF (Harlan Bioproducts, Indianapolis, IN). For CGNs, two different media were used: K5+S (Basal Medium Eagle's [Life Technologies] containing 10% dialyzed fetal bovine serum, 5 mM KCl, 100 U/ml penicillin, and 100 μg/ml streptomycin) and K25+S (K5+S plus 20 mM KCl). The breeding and genotyping of *Bcl-2*-overexpressing, *Bax*-deficient, and *Bim*-deficient mice have been described previously (Martinou et al., 1994; Knudson et al., 1995; Deckwerth et al., 1996; Bouillet et al., 1999).

Sympathetic Neuronal Cultures

Primary cultures of sympathetic neurons were established from the superior cervical ganglia (SCG) of neonatal mice or rats by using previously described methods (Johnson and Argiro, 1983; Deckwerth et al., 1996; Easton et al., 1997). Neurons were grown in AM50 for ~5 DIV and then either maintained in AM50 or treated as follows. For NGF deprivation, cultures were rinsed with AM0, followed by the addition of AM0 containing goat anti-mouse 2.5S NGF neutralizing antiserum (anti-NGF; Ruit et al., 1990). For NGF deprivation in the presence of various reagents, 1 μg/ml CHX, 0.1 μg/ml ActD, 40 mM KCl, 400 μM CPT-cAMP, 250 nM CEP-1347, or 50 μM BAF was added to AM0 containing anti-NGF. In certain experiments, DIV5 neurons were maintained in AM50 in the presence of 50 μM LY294002, 250 nM CEP-1347, 100 nM STS, or 1 μM STS for the indicated times.

CGN Cultures

Primary cultures of CGNs were obtained as described previously (Miller and Johnson, 1996; Miller et al., 1997a). Briefly, P7 cerebella were dissected, trypsinized, triturated, and plated into K25+S medium at a density of 2.3×10^5 cells/cm^2 in four-well dishes (Nunc, Naperville, IL) coated with 0.1 mg/ml poly-L-lysine. To reduce the number of nonneuronal cells, 3.3 μg/ml aphidicolin was added to the medium 36 hr after plating. At DIV7, CGNs were either maintained in K25+S or switched to K5+S after washing once with the respective medium. Cell viability was then assessed at the indicated times by taking photomicrographs of representative fields of cells labeled

with calcein AM (Molecular Probes, Eugene, OR) as described previously (Miller and Johnson, 1996).

RT–PCR Analysis

RT–PCR analysis of neuronal cultures has been described previously (Estus et al., 1994; Miller and Johnson, 1996). Briefly, mRNA was isolated from NGF-maintained DIV7 sympathetic neurons, K25+S-maintained DIV7 CGNs, and P1 DRG neurons by using an oligo-dT cellulose mRNA purification kit (QuickPrep Micro kit; Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Half of the mRNA was converted into cDNA by reverse transcription by using Moloney murine leukemia virus reverse transcriptase (Superscript, Life Technologies) and random hexamers (16 μM) as primers. For PCR analysis, 1% of the cDNA was used in a 50 μl PCR reaction; half of the PCR reaction was separated on a 10% polyacrylamide gel, and the PCR product was visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Sciatic Nerve Axotomy

Axotomies were performed on neonatal rats anesthetized with methofluorane (Schering-Plough Animal Health, Union, NJ) as described previously (Yip et al., 1984). Briefly, the right sciatic nerve of each rat was transected near the tendon of the obturator internus; the left sciatic nerve served as an unlesioned control. The wound was closed with Nexaband (Veterinary Products Labs, Phoenix, AZ), and the success of the surgery was demonstrated by partial paralysis of the operated limb. After 24 hr, the L4 and L5 DRGs from both sides were dissected, homogenized in lysis buffer, and evaluated by Western blotting.

Subcellular Fractionation, Alkali Extraction, and Immunoblotting

Fractionation, extraction, and immunoblotting of sympathetic neurons were performed as described previously (Putcha et al., 2000; Tsui-Pierchala et al., 2000). Primary antibodies included: α-tubulin (1:100000; Sigma); Akt (1:1000; New England Biolabs, Beverly, MA); Bim clones 5E5 and 14A8 (1:1000); Bim/Bod (1:1000; StressGen, Victoria, British Columbia); cyt c (1:1000; Pharmingen, San Diego, CA); cytochrome oxidase subunit IV (COX4; Molecular Probes); lactate dehydrogenase (LDH; 1:1000; Rockland Immunochemicals, Gilbertsville, PA); phospho-Akt S473 (P-Akt; 1:1000; New England Biolabs); and phospho-c-Jun S63 (P-Jun; 1:200; New England Biolabs). Appropriate horseradish peroxidase–conjugated (HRP-conjugated) secondary antibodies (New England Biolabs and Jackson ImmunoResearch) were diluted to 1:5000−1:10000.

Cell Counts

Commitment Point Experiments

Sympathetic neurons from *Bim*$^{-/-}$, *Bim*$^{+/-}$, and *Bim*$^{+/+}$ mice were deprived of NGF as described above. At various times after deprivation, cultures were rinsed with AM0 and then incubated in AM50 for 5−7 days. Neurons were then counted after being washed with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, washed again with PBS, and stained with toluidine blue as described previously (Deshmukh et al., 1996).

P-Jun and Cyt c Immunocytochemistry

Neuronal cultures were immunostained as described previously (Putcha et al., 1999). Briefly, sympathetic neurons that had been maintained in NGF for ~5 DIV in AM50 were deprived of NGF in the presence of the caspase inhibitor BAF (as described above). At various times after deprivation, cultures were fixed and immunostained with anti-P-Jun (1:200; NEB) or anti-cyt c (1:1000; Pharmingen) antibodies. The respective secondary antibodies were Cy3-conjugated donkey anti-rabbit and anti-mouse IgG (1.5 mg/ml; Jackson ImmunoResearch, West Grove, PA), respectively, diluted 1:400. For each time point, the number of cells that had acquired a nuclear staining pattern for P-Jun or had lost the punctate staining pattern for cyt c was determined by a naïve observer from a random sampling of 100–200 cells. All experiments were conducted in the presence of BAF to prevent any cell loss that would otherwise affect the counts.

TUNEL

Tyramide signal amplification (TSA) of TUNEL staining was performed as described previously (Shindler et al., 1997). Briefly, fresh-frozen sections were incubated with 0.5% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 10 min at room temperature, washed, and incubated in TdT buffer (Roche Molecular Biochemicals, Indianapolis, IN). The buffer was then decanted, the TdT reaction mix containing TdT-digoxygenin was applied, and the sections were incubated for 60 min at 37°C. The reaction was stopped, and sections were washed and then incubated with anti-digoxygenin antibodies diluted 1:1000 overnight at 4°C. Sections were washed and visualized by using direct TSA (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. A random, systematic sample of sections was examined by fluorescence microscopy. Only neurons, which were identified as cells containing large, round nuclei (contrasted with the flattened, elliptical nuclei of nonneuronal cells) were counted by a naïve observer.

Statistics

When indicated, statistical significance was determined by a Student's t test or by a Mann-Whitney rank sum test for parametric and nonparametric data, respectively.

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Note Added in Proof

Since the acceptance of this manuscript, we have become aware of two reports describing BIM induction during trophic factor deprivation in two different models of nonneuronal cell death (Dijkers et al., Curr. Biol. *10*(*19*): 1201–1204, 2000; Shinjyo et al., Mol. Cell. Biol. *21*(*3*): 854–864, 2001). Although the reports do not address whether upregulation of BIM expression is required for cell death in these paradigms, the findings suggest that BIM may represent a common intracellular sensor for trophic factor deprivation, at least in both neurons and hematopoietic cells. Interestingly, other putatively apoptotic stimuli do not induce BIM in these hematopoietic cell lines, consistent with the idea that BIM may be required only for certain cell death pathways.